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14. ABSTRACT

More than 90% of deaths caused by breast cancer are attributed to metastatic disease. However, the exact molecular mechanism of tumor metastasis is still poorly understood. It has been well recognized that only a fraction of cells in the primary tumor eventually metastasizes to the distant organs; however, the origin and nature of these cells are still unclear. The purpose of this project is to test our novel hypothesis that metastatic cells originate from a distinct tumor cell population which has both stem-like properties and an invasive ability. We have successfully isolated the cell population (CD24-/ CD44+/ ESA+) that has tumor initiating ability as well as metastatic capability. The expression profile analysis revealed that the HAS2 gene plays a critical role in the process of bone metastasis of CSCs, which was also strongly supported by our results of in vivo experiment. Interestingly, small molecule HAS2 inhibitor, 4MU, was shown to significantly suppress the CSC-induced bone metastasis. Therefore, our results open a possibility of using 4MU for the treatment of metastatic bone disease.

15. SUBJECT TERMS

Breast cancer, cancer stem cell, metastasis

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INTRODUCTION

More than 90% of deaths caused by breast cancer are attributed to metastatic disease (1). However, the exact molecular mechanism of tumor metastasis is still poorly understood. It is well recognized that only a fraction of cells in the primary tumor eventually metastasizes to the distant organs; however, the origin and nature of these cells are still unclear (2). The purpose of this project is to test our novel hypothesis that metastatic cells originate from a distinct tumor cell population which has both stem-like properties and an invasive ability. The overall objective of our project is to identify metastatic stem cells of breast cancer and define basic characteristics of these cells. To test our hypothesis, we will (i) isolate stem-cell population from non-metastatic and metastatic cells of a pair of syngenic breast tumor cell lines, and test their metastatic ability in an animal model, and (ii) examine their gene expression profiles by microarray analysis and verify the results in tumor stem cell of human breast cancer specimens.

BODY

Task 1. To isolate stem-cell population of non-metastatic and metastatic cells from a pair of syngenic breast tumor cell lines and test their metastatic ability in an animal model.

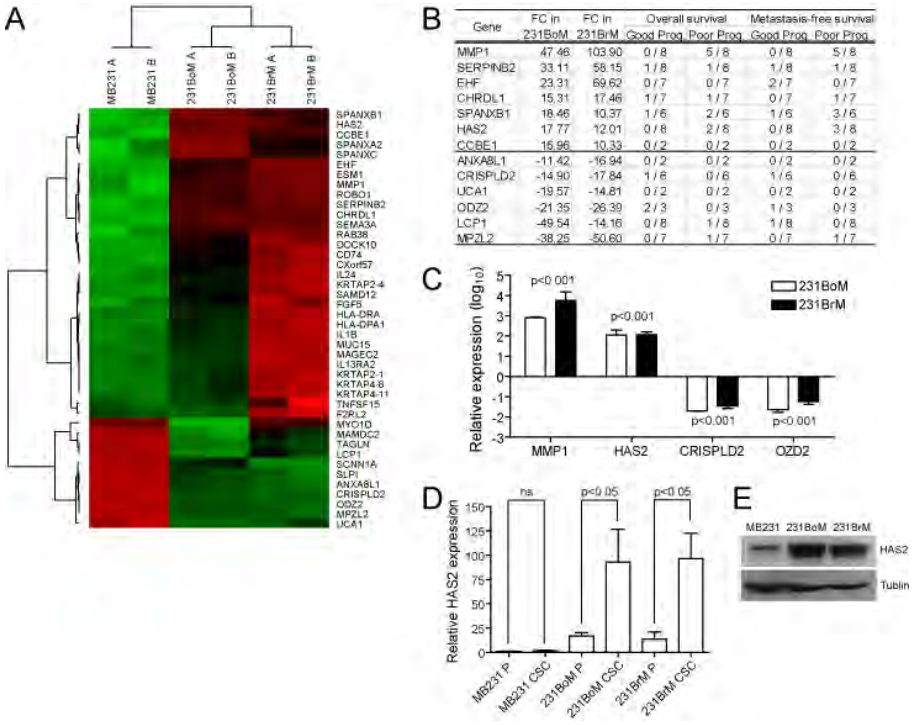
(a) "Label" MDA-MB231 and BoM-1833 cells with the luciferase gene with either wild type or mutant.
(b) Isolate tumor stem cells (CD24-, CD44+ and ESA+) from both cell lines.
(c) Test their metastatic ability in DOD-SCID mice.

To accomplish Task 1, we have labeled MB231, 231BoM and 231BrM cell lines using luciferase-expressing lentivirus followed by isolation of CD24-/ CD44+/ ESA+ population by the magnetic bead sorting using MACS Separator (Miltenyi Biotec). The latter two cell lines were originally isolated by Massague's group from metastasized tumors in bones and brain after injecting MB231 into animals. They are highly metastatic to bones and brain, respectively, when they are transplanted into immunodeficient mice (3, 4). Flow cytometric analysis of these cell lines using previously identified surface markers for CSCs, CD24, CD44 and EpCAM (ESA) (5,6) indicate that these cell lines contain a minor population (2-6%) of CD24-/CD44+/EpCAM+ cells. To examine the tumor initiating ability of these cells, we transplanted various doses of CSCs into nude mice and monitored the tumor formation and metastasis. The results of this limiting dilution analysis clearly indicate that the CD24-/ CD44+/ ESA+ population had significantly stronger ability of tumor initiation and metastasis compared to the non-CSC population or parent cells (Table I). We then used these cells for Task2. **Therefore, Task 1 is completed.**

Table 1

Strain	Population	Number of tumors/number of injections: Cells per injection 10,000	1,000	100	10	Tumor-initiating cell frequency (95% cenfidence interval)	
MDA-MB-231	Unsorted	2/4	1/4	0/2		1/10,720	(1/3,203-1/35,879)
	Stem cells	6/6	5/6	2/6		1/448**/$$	(1/183-1/1,097)
	Non-stem cells	1/2	0/2	0/2		1/16,705	(1/2,356-1/118,284)
231BoM-1833	Unsorted	6/6	6/7	3/5	0/3	1/334	(1/140-1/844)
	Stem cells	5/5	11/11	9/11	6/11	1/37**/$$	(1/19-1/72)
	Non-stem cells		2/4	0/4	0/4	1/1,671	(1/419-1/6,668)
231BrM-2a	Unsorted		4/4	1/4	0/4	1/277	(1/86-1/895)
	Stem cells		4/4	5/6	2/6	1/45*/$$	(1/19-1/110)
	Non-stem cells		5/6	1/6	0/6	1/569	(1/236-1/1374)

*: P<0.05 (Unsorted vs stem cells)
**: P<0.0001 (Unsorted vs stem cells)
$$: P<0.0001 (Non-stem cells vs stem cells)



Gene	FC in 231BoM	FC in 231BrM	Overall survival Good Prog.	Overall survival Poor Prog.	Metastasis-free survival Good Prog.	Metastasis-free survival Poor Prog.
MMP1	47.46	103.90	0 / 8	5 / 8	0 / 8	5 / 8
SERPINB2	33.11	58.15	1 / 8	1 / 8	1 / 8	1 / 8
EHF	23.31	69.62	0 / 7	0 / 7	2 / 7	0 / 7
CHRDL1	15.31	17.46	1 / 7	1 / 7	0 / 7	1 / 7
SPANXB1	18.46	10.37	1 / 6	2 / 6	1 / 6	3 / 6
HAS2	17.77	12.01	0 / 8	2 / 8	0 / 8	3 / 8
CCBE1	16.96	10.33	0 / 2	0 / 2	0 / 2	0 / 2
ANXA8L1	-11.42	-16.94	0 / 2	0 / 2	0 / 2	0 / 2
CRISPLD2	-14.90	-17.84	1 / 8	0 / 8	1 / 6	0 / 6
UCA1	-19.57	-14.81	0 / 2	0 / 2	0 / 2	0 / 2
OZD2	-21.35	-26.39	2 / 3	0 / 3	1 / 3	0 / 3
LCP1	-49.54	-14.16	0 / 8	1 / 8	1 / 8	0 / 8
MPZL2	-38.25	-50.60	0 / 7	1 / 7	0 / 7	1 / 7

Figure 1. HAS2 gene is upregulated in cancer stem-like cells from metastatic breast cancer cells. (A) CSCs from MB231, 231BoM and 231BrM were prepared, and their RNAs were subjected to expression array analysis. A heatmap was generated for the genes that were significantly up- or down-regulated at least 10 times in CSCs among 231BoM, 231BrM and MB231. (B) Overall- or metastasis/relapse-free survival of breast cancer patients who have altered expression of these genes were examined. A number of cohort data which showed significant difference was recorded. The individual cohort data and the result are listed in Table S1 and S2, respectively. FC indicates fold-change. (C) The expression of *MMP1*, *HAS2*, *CRISPLD2* and *OZD2* were examined by qRT-PCR for CSCs prepared from these three cell lines. The degree of the expression of each gene was expressed by log10-scaled fold-changes and the expression level of CSCs of MB231 was set as 0 in log10-scale. Data are represented as mean±SEM (n=3). (D) *HAS2* expressions in both parental cells and CSCs from each cell line were measured by qRT-PCR. P; Parental cells, CSC; stem cell populations of the cell lines. Data are represented as mean±SEM (n=3). (E) Western blotting for HAS2 protein in CSCs was shown.

Task 2. To examine their gene expression profiles by microarray analysis and verify the results in tumor stem cell of human breast cancer specimens.

(a) Analyze expression profile of non-metastatic and metastatic stem cells by Affymetrix microarray.
(b) Establish "metastatic signature" of stem cell by data analysis.
(c) Isolate tumor stem cells from both primary and lymph node metastatic lesions and examine the expression of "metastatic signature" genes in these cells.

We then performed global expression profile analysis for these CSCs using the Affymetrix expression array. A comparison of transcriptional profiles uncovered 219 genes whose mRNA were up-regulated at least 4-fold in CSC of highly metastatic cell lines (231BoM and 231BrM) compared to the CSC of MB231. On the other hand, we found that 42 genes were down-regulated in CSC of 231BoM and 231BrM more than 4 times. When this threshold was increased to 10-fold, there were 31 up-regulated

and 11 down-regulated genes, which was shown as a heat-map in Figure 1A. To examine the clinical relevance of the 42 genes that were listed in Figure 1A, we first chose 7 genes including *MMP1*, *SERPINB2*, *EHF*, *CHRDL1*, *SPANXB1*, *HAS2* and *CCBE1* that were highly over-expressed in CSC of

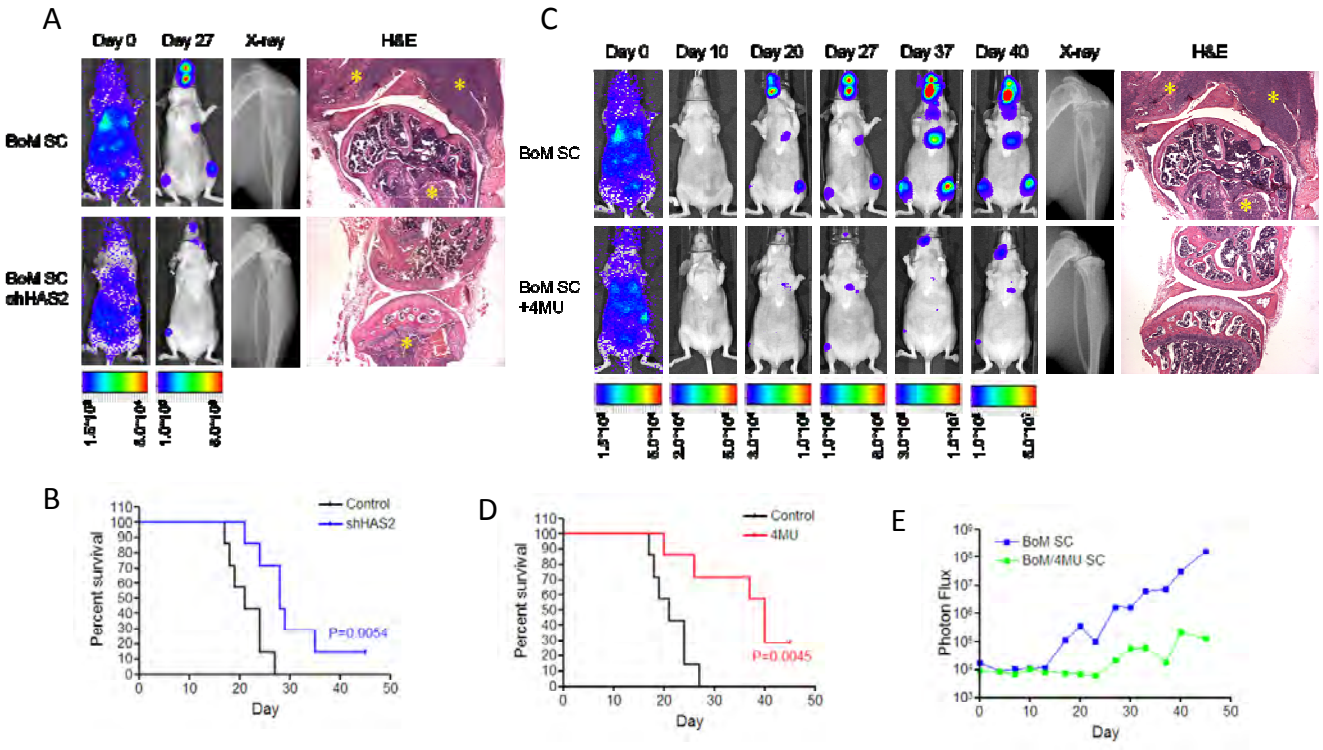


Figure 2. HAS2 enhances metastasis in vivo
(A) CSCs were isolated from 231BoM or 231BoM-shHAS2 and 5 x 104 cells were intracardially injected into nude mice. Images are bioluminescent, radiographic, and H&E analysis of bone lesions from representative mice in each group at the indicated times after CSCs injection. The osteolytic lesions in the X-ray image are indicated by arrows. Tumors appearing in H&E stained photos are indicated by asterisk. (B) Kaplan-Meier analysis for metastasis-free survival of these animals were performed (n=9-10 per group). (C) CSCs of 231BoM were intracardially injected into the nude mice as described in (A). The animals were then fed with or without 4-MU (400 mg/kg/day, every day for 45 days). The metastatic tumor growth in tibia was measured as described in (A). (D) Kaplan-Meier analysis for metastasis-free survival of these animals was performed (n=9-10 per group). (E) The metastatic growth of tumors in tibia with or without the treatment of 4-MU was monitored by Xenogen bioimager and the median value of the bioluminescence was plotted. (n=9-10 per group).

metastatic cells. We also selected 6 genes including *ANXA8L1*, *CRISPLD2*, *UCA1*, *ODZ2*, *LCP1* and *MPZL2* that were found to be significantly down-regulated in CSC of these cell lines. We then examined the relationship between the expression of these genes and overall- and metastasis-free survival of breast cancer patients using multiple existing data base. As shown in Figure 2B, among the seven most up-regulated genes of our array analysis, two genes (*MMP1* and *HAS2*) were positively correlated with poor overall- and metastasis-free survival. On the other hand, the down-regulation of two genes (*CRISPLD2* and *ODZ2*) was significantly correlated with good overall- and metastasis-free survival. We also examined the expression of these genes by qRT-PCR and confirmed that the expressions of the genes were indeed significantly altered in CSCs from 231BoM and 231BrM compared to that of MB231 (Figure 1C). Notably, recent evidence suggests that the *HAS2* expression is significantly correlated with tumorigenicity and tumor progression, and therefore, HAS2 is of considerable interest for further study. When we examined the expression of *HAS2* in both CSC and

parental cells by RT-PCR, *HAS2* gene expression was indeed shown to be specifically overexpressed in isolated CSCs from metastatic variant cell lines and these results were further confirmed by Western blot (Figure 1E and F).

To further examine the role of *HAS2* in tumor metastasis *in vivo*, we prepared CSCs from 231BoM which was "labeled" with the luciferase gene and implanted them into nude mice by intra-cardiac injection. The tumor metastasis in the bones was monitored by BLI (Xengen). Luminescence from the injected cells was distributed all over the bodies of the mice immediately after injection, confirming the successful cardiac injection. At day 27, metastatic tumors were clearly visible in tibial bones and jaws (Fig. 2A). On the other hand, introduction of shRNA to *HAS2* significantly suppressed the metastatic spread of tumor cells. We also examined osteolytic bone lesions by X-ray imaging and found that CSCs from 231BoM generated large osteolytic bone lesions at 27 days after injection; however, introduction of shRNA to *HAS2* significantly decreased osteolytic lesions in bones. We also examined the metastasized tumor in the bone by microdissecting the bone lesion followed by H&E staining. The bone lesions generated by injection of 231BoM CSCs were strongly osteolytic and extended to the outside of bones, while the bone lesions generated by 231BoM CSC carrying shRNA for *HAS2* were much less extensive and remained in the bone. As shown in Figure 2B, mice inoculated with CSCs of 231BoM which carried shRNA for *HAS2* significantly improved the metastasis-free survival rate, implying that HAS2 plays a critical role in metastasis of CSC. Next, we also investigated the effect of HAS 2 inhibitor, 4-MU, on the metastatic ability of CSCs to the bones using the same mouse model. We intracardially injected CSCs of 231BoM to the mice followed by daily oral administered 4-MU. We found that administration of 4-MU significantly suppressed the incidence of metastasis of CSC to the bones and also significantly improved metastasis-free survival of the animals (Fig. 2C-E). Consequently, 4-MU also protected mice from osteolytic damage. The 4-MU treatment did not affect the body weight of these mice and did not show noticeable toxic effects. These results indicate that inhibition of *HAS2* markedly reduces the risk of bone metastasis in breast cancer and that 4-MU may be used as a potential anti-metastatic drug for the treatment of cancer patients.

Therefore, we have accomplished Task 2 (a) and (b). However, Task 2(c) is still underway.

KEY RESEARCH ACCOMPLISHMENTS

1. We have successfully isolated tumor stem cell population from breast metastatic cell lines and tested their stemness *in vivo*.
2. Our Affymetrix expression array analysis revealed that there are 31 up-regulated and 11 down-regulated genes in cancer stem cells.
3. We found that the Has2 gene is significantly up-regulated in metastatic CSCs.
4. Our *in vivo* analysis indicated that knockdown of the HAS2 gene significantly reduced CSC-induced metastasis in our animal model of breast cancer metastasis.
5. The small molecule inhibitor of HAS2, 4MU, significantly reduced the CSC-induced bone metastasis in animals, suggesting that this compound can be used for a preventive and therapeutic purpose in metastatic bone disease of breast cancer.

REPORTABLE OUTCOMES

Peer reviewed publications

1. Hiroshi Okuda, Aya Kobayashi, Bo Xia, Misako Watabe, Sudha K Pai, Shigeru Hirota, Fei Xing, Wen Liu, Puspa R Pandey, Koji Fukuda, Vishnu Modur, Arnab Ghosh, Andrew Wilber and

Kounosuke Watabe. HAS2 promotes metastasis by stimulating interaction of breast cancer stem cells with macrophage and stromal cells in the bone. Cancer Research, pending revision

Abstract/presentation

1. Hiroshi Okuda, Aya Kobayashi, Misako Watabe, Sudha K. Pai, Wen Liu, Fei Xing, Puspa R. Pandey, Koji Fukuda, Megumi Iiizumi, Kounosuke Watabe. Hyaluronan synthase 2 promotes tumor progression by stimulating interaction of cancer stem-like cells with tumor associated macrophage and stromal cells in the bone. 2011 Annual meeting of American Association for Cancer Research. Orlando FL

Employment

1. Dr. Hiroshi Okuda (postdoctoral fellow) has been partly supported by the current grant.

CONCLUSIONS

Tumor metastasis is an extremely inefficient process and only a fraction of cells in the primary tumor can successfully establish metastatic colonization. These cells by definition have a stem-like ability, but they also need to have a capability of metastasizing to other organs. We have successfully isolated the cell population (CD24-/ CD44+/ ESA+) that has tumor initiating ability as well as metastatic capability. The expression profile analysis revealed that the HAS2 gene plays a critical role in the process of bone metastasis of CSCs, which was also strongly supported by our results of *in vivo* experiment. Interestingly, small molecule HAS2 inhibitor, 4MU, was shown to significantly suppress the CSC-induced bone metastasis. Therefore, our results open a possibility of using 4MU for the treatment of metastatic bone disease.

So what?

Our finding revealed the existence of metastatic CSCs that have both tumor initiating ability and metastatic capability. This suggests that the metastatic CSCs are critical target for preventing and treating metastatic disease of breast cancer. Our data also indicate that one of the key genes, HAS2, which plays a critical role in CSCs, may be a potential therapeutic target. We indeed showed that a small molecule HAS2 inhibitor can significantly suppress the CSC-induced metastasis. Considering the non-toxicity of 4MU, it may open a new opportunity to develop an anti-metastasis drug for breast cancer.

We believe that the identification of metastatic stem cell population offers a paradigm shift in this research field and also provides with rationale of critical target cells for treating patients with metastatic disease which is still the major reason why patients succumb to this devastating disease.

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